REMARKS

The Examiner is thanked for the due consideration given the application.

Claims 20-38 are pending in the application. No new matter is believed to be added to the application by this response.

The Official Action has restricted the claims of the application into the following Groups:

Group I, claims 20-29 and 36, drawn to an enzymatically-active glutamine;

Group II, claims 30-32, drawn to a nucleic acid, recombinant vector and vector;

Group III, claims 33-35, drawn to a purification process for a protein;

Group IV, claim 37, drawn to a method for screening of compounds modifying the activity of a protein; and

Group V, claim 38, drawn to a method of screening compounds useful for the treatment of diabetes.

Group I, claims 20-29 and 36 is elected with traverse.

The Official Action additionally requires election of purported species selected from SEQ ID NOs:1-12.

SEQ ID NO 8 is elected with traverse.

As is set forth in MPEP 803, there are two criteria for a proper requirement for restriction between patentably distinct inventions:

(A) The inventions must be independent or distinct as claimed; **and**

(B) There would be a serious burden on the Examiner if restriction is not required.

In this case the technologies of the enzymatically-active glutamine of Group I, the nucleic acid, recombinant vector and vector of Group II, the purification process of Group III and the screening methods of Groups IV and V are so intimately interrelated that no undue burden is placed upon the Examiner to examine all the groups (and species) on the merits.

As evidence thereof, it is noted that the Official Action has cited the prior art references of CHANG et al. (*J. Biol. Chem.* 2000) and FERGUSON et al. (*Protein Sci.* 1998) to assert that Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT rule 13.2, they lack the same of corresponding special technical features, where "special technical features" is defined to mean those technical features that define a contribution over the prior art.

By this, the Office has already performed consideration and/or search based upon the prior art. There is thus clearly no additional undue burden to continue to examine the entire present invention on the merits.

Nonetheless, the Official Action asserts that the combination of CHANG et al. and FERGUSON et al. would cause one of skill in the art to provide a GFAT protein containing an

internal purification 6xHis Tag, inserted between two amino acids.

CHANG et al. pertain to GFAT protein fused in its N-terminus to allow a purification of the enzyme. This tagged protein however displays a low GFAT activity, less than the natural enzyme.

FERGUSON et al. pertain to a protein from *E. coli*, FhuA, wherein a 6xHis Tag has been inserted between two amino acids located on surface-exposed loop of the protein.

GFAT is a dimeric (bacteria) or tetrameric (human) enzyme which belongs to the N-terminal nucleophile (NtN) hydrolase family. Each enzyme subunit is organized into two domains, the glutaminase domain responsible for hydrolysis of L-glutamine (Gln) into L-glutamate (Glu) and the isomerase domain responsible for ketose/aldose isomerization of the RR'C=NH aminosugar phosphate adduct. These two domains are connected by an intramolecular channel responsible for the transfer of ammonia.

GFAT are cytosolic proteins which are found in all organisms from bacteria to mammals.

FhuA, the receptor for ferrichrome-iron in *E. coli* mediates the active transport of ferric siderophores through the outer membrane of Gram-negative bacteria. It is representative of a wider family of bacterial outer membrane active transporters.

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With respect to the assertion in the Official Action, the two proteins that are proposed to combine to provide the 6xHisTagged GFAT of the present invention, they are

1- different in their 3-dimensional structure,

2- different in their function,

3- different in their cellular localization, and

4- expressed in a different organism.

It is common practice in the biological sciences to compare proteins in order to provide new techniques/strategies and methods for a better understanding of their function.

However, the common practice in the art is to compare proteins with similar functions, or structures, and preferably proteins expressed in the same type of organisms and/or organs.

So, a person with an ordinary skill would combine results from an article demonstrating that a N-terminus tag of its preferred protein induce a decrease of its activity, with the results of an article demonstrating that an internal tag of a *closely similar protein* maintain its 3D-structure and function.

In contrast, a person with an ordinary skill would fail to combine the subject matter of CHANG et al. and FERGUSON et al.

As argued in the Official Action, FERGUSON et al. disclose a functional FhuA protein (page 1637, end of the second paragraph) where a 6xHis tag has been inserted after the amino acid at position 405. As mentioned in this document, amino acid 405 of FhuA is located in a surface-exposed loop of the protein.

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At the filing date of the present application, the inventors knew that *E. coli* GlmS, the bacterial GFAT homologue, was sensitive to peptidic-cleavage by chymotrypsine, after the amino acid 240 (see OBMOLOVA et al., *J. Mol. Biol.* 1994, 242, pp 703-705, page 703 second paragraph, and see DENISOT et al., *Archives of Biochemistry and Biophysics*, 288(1), pp 225-230, all the document; attached to this paper).

These data strongly suggested that position 240 in the GlmS protein, and *per se* the homologous position L313 in the human GFAT1, was contained in a surface-exposed loop.

However, the inventors have demonstrated that GlmS protein containing a 6xHis tag after the amino acid 240 inhibits the protein activity, such as the GST-GFAT disclosed by CHANG et al. The results are presented in the following table:

Table 1: Synthase activity of wild type GlmS and of its affinity purified fusion mutants.

Enzyme Synthase	Activity (U/mg)	Comments
WTGlmS	8 ± 0.3	Natural protein
GST-Nt-GlmS	0.007 ± 0.001	CHANG et al. protein
His6-225-GlmS	8 ± 0.4	Invention
His6-240-GlmS	**0.08 ± 0.02**	**His tag in chimotrypsine site**

Therefore, if the subject matter of FERGUSON et al. and CHANG et al. is combined, as proposed by the Official Action, the

"compiled result" would disclose a 6xHis Tagged GFAT, where the tag is inserted after the amino acid homologous to the position 240 of the GlmS protein, which conserves its 3D structure and its function. But, this GFAT-tagged enzyme has a low specific activity.

Therefore, the combination of CHANG et al. and FERGUSON et al. would provide a *non-functional* 6xHis tag GFAT protein, which clearly does not correspond to the protein of the present invention.

To date, there are only few examples of proteins with an internal tag, compared to N-ter or C-ter tagged protein, probably because it is not evident to find the correct amino acid locations that allow the conservation of 3D-structure and activity.

Then, concerning GFAT protein, a skilled person would not find, with reasonable expectation of success, i.e., without testing all the possibilities, the amino acids sequence wherein to insert a 6xHis tag to provide a GFAT protein that retain its structure and its activity.

As a consequence, for all the above-mentioned reasons, the present invention should be considered as a single general inventive concept, since the technical features of the present application are not obvious in view of the combination of the cited prior art.

Accordingly rejoinder and examination of all the claims and sequences of the present invention are respectfully requested.
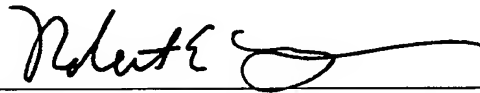
Conclusion

Early and favorable prosecution on the merits is respectfully requested.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

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APPENDIX:

The Appendix includes the following item(s):

- Two articles:

OBMOLOVA et al., *J. Mol. Biol.* 1994, 242, pp. 703-705, and

DENISOT et al., *Archives of Biochemistry and Biophysics*, 288(1), pp. 225-230.

Crystallization and Preliminary X-ray Analysis of the Two Domains of Glucosamine-6-phosphate Synthase from *Escherichia coli*

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The glutamine amidohydrolase and fructose 6-phosphate binding domains of glucosamine-6-phosphate synthase from *Escherichia coli* have been overexpressed, purified and crystallized for X-ray diffraction analysis. The crystals of the glutamine amidohydrolase domain belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 70.4$ Å, $b = 82.5$ Å, $c = 86.1$ Å, with two molecules in the asymmetric unit, and diffract to 1.9 Å resolution. The native Patterson indicated pseudo c-face centering of the unit cell. The fructose 6-phosphate binding domain was crystallized in the hexagonal space group $P6_1$ or $P6_5$ with cell dimensions $a = b = 63.5$ Å, $c = 334.3$ Å and with two molecules in the asymmetric unit. Diffraction data to 2.6 Å resolution have been collected.

Keywords: glucosamine-6-phosphate synthase; *Escherichia coli*; crystallization; X-ray analysis

Glucosamine 6-phosphate synthase catalyzes the first reaction in hexosamine biosynthesis (Badet *et al.*, 1987). It belongs to the F-type group of the glutamine-dependent amidotransferase family of enzymes (Zalkin, 1993), which utilize the glutamine amide nitrogen in the biosynthesis of phosphoribosylamine (phosphoribosyl pyrophosphate amido transferase; Tso *et al.*, 1982), glutamate (glutamate synthase; Miller & Stadtman, 1972) or asparagine (asparagine synthetase: Scofield *et al.*, 1990). The product of the reaction with fructose 6-phosphate, glucosamine 6-phosphate, undergoes sequential transformations leading to the formation of uridine diphospho-*N*-acetylglucosamine (UDPGlcNAc†), which is the unique precursor of all amino sugar-containing macromolecules. The inhibition of the microbial or human enzyme is believed to have important implications in antibacterial/antifungal therapy (Andruszkiewicz *et al.*, 1990) or treatment of diabetes (Traxinger & Marshall, 1992).

The bacterial enzyme comprises two domains that can be separated by limited chymotryptic proteolysis (Denisot *et al.*, 1991). The glutamine binding domain encompassing residues 1 to 240 has the same capacity to hydrolyze glutamine (and the corresponding *p*-nitroanilide derivative) into glutamate as the native protein. The amino acid sequence of the glutamine binding domain is highly conserved among members of the F-type group of amidotransferases. The 368-residue carboxy-terminal domain retains the ability to bind fructose 6-phosphate.

While Cys1 has been shown to be essential for the activity of the native enzyme (Badet *et al.*, 1987), the mechanism of nitrogen transfer from glutamine to the acceptor is still a matter of controversy (Zalkin, 1993; Richards & Schuster, 1992). Solving the three-dimensional structure of the enzyme would certainly be helpful for understanding this puzzling piece of enzyme chemistry.

The complexity of the enzyme structure and the reaction catalyzed prompted us to undertake the analysis of each domain separately. The two domains were overexpressed by manipulating the phagemid pMA1, which was obtained by insertion of the 2·6 kb *Sac*I-*Bam*HI fragment of plasmid pGM10A (Dutka-Malen *et al.*, 1988) into pUC118 as follows. Two stop codons followed by the *Nde*I site were inserted at the position corresponding to Tyr240 by site-directed mutagenesis to create the recombinant phagemid pMA100. The 1·3 kb *Nde*I-*Bam*HI fragment was purified and ligated into phage T7-7 at the corresponding sites. The phagemid pMA200 was

generated by subcloning the *Xba*I-*Bam*HI fragment containing the ribosome binding site of T7-7, the initiation site generated by *Nde*I, and the DNA encoding the glucosamine 6-phosphate synthase C-terminal domain, into pUC119. Both phagemids pMA100 and pMA200 ensured overexpression of the corresponding domains using *Escherichia coli* HB101 as host. The proteins were purified to homogeneity using a combination of ion-exchange and gel-filtration chromatography.

The glutamine amidohydrolase domain was crystallized by vapor diffusion in hanging drops at 4 °C. The protein solution (3 μl; 15 mg/ml) in 0·1 M cacodylate buffer, (pH 6·5) with 0·2% β-octyl-glucoside were mixed with 3 μl of the reservoir solution, which contained 1 M sodium acetate and 20% polyethylene glycol 4000. Within two weeks, the crystals reached a size of 0·5 mm × 0·5 mm × 0·2 mm. They belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 70·4$ Å, $b = 82·5$ Å, $c = 86·1$ Å. Assuming two protein molecules in the asymmetric unit gives a solvent content of 48% and a V_M of 2·4 Å^3/Da (Matthews, 1968). Diffraction data were collected from one of these crystals using synchrotron beam line X11 (EMBL, Hamburg) and a MAR Research imaging plate scanner. A total of 170,000 independent measurements were merged to obtain 36,993 unique reflections ($R_{merge} = 5·4\%$) to 1·9 Å resolution, which constitute 99% of the theoretically possible data.

Self-rotation studies on these data showed no significant peaks and suggested that the two protein molecules were in the same orientation in the asymmetric unit. The presence of a strong non-origin peak, at approximately 1/2, 1/2, 0, in the native Patterson synthesis confirmed this (Figure 1). The diffraction pattern does indeed show that these crystals exhibit pseudo *c*-face centering as would be expected if one molecule was related to the other by a purely translational symmetry of 1/2, 1/2, 0.

The fructose 6-phosphate binding domain was crystallized in hanging drops at 4 °C in the presence and absence of the substrate fructose 6-phosphate. Protein solution (10 mg/ml) in 0·1 M Hepes buffer (pH 7·0) was mixed with the equal amount of the reservoir solution containing 30% (NH$_4$)$_2$SO$_4$, 28% sodium formate, and 1·4 M Li$_2$SO$_4$. The hexagonal bipyramidal crystals reached a size of 0·6 mm × 0·3 mm × 0·3 mm and diffracted to 2·2 Å. They belong to the space group $P6_1$ or its enantiomorph $P6_5$ with cell dimensions $a = b = 63·5$ Å, $c = 334·3$ Å. There are probably two protein molecules in the asymmetric unit giving a V_M of 2·4 Å^3/Da and a solvent content of 49% (Matthews, 1968). Diffraction data to 2·6 Å resolution were collected on beam line X11 ($\lambda = 0·92$ Å) using a MAR Research 300 mm imaging plate scanner at a distance of 390 mm from the crystal. A total of 108,620 independent measurements were merged to obtain 23,992 unique reflections ($R_{merge} = 5·4\%$), which constitute 99% of the theoretically possible data. A search for heavy-atom derivatives is in progress.
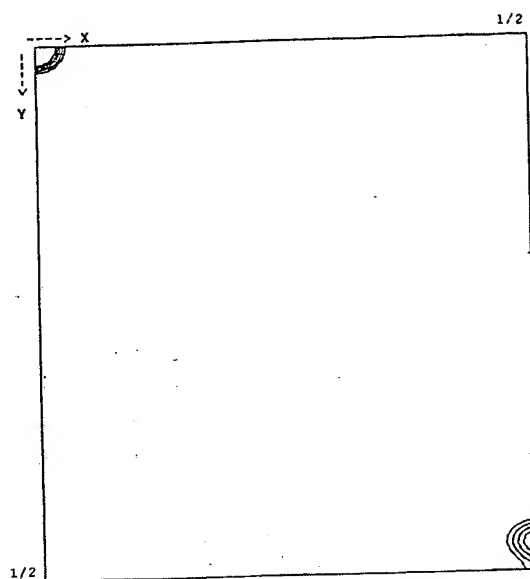

Figure 1. Section $z = 0$ of the native Patterson of the glutamine amidohydrolase domain crystals. The map was calculated using all data between 12 and 3 Å. The origin peak has been scaled to a value of 100 and the map has been contoured from 10 to 50 in steps of 10.

Small crystals of the intact protein glucosamine 6-phosphate synthase were obtained under conditions similar to those for the fructose 6-phosphate binding domain: crystallization solution contained 10 mg/ml protein, 1 M LiCl, and 3% PEG 4000 in 0·1 M Hepes buffer (pH 7·0). The crystals belong to the monoclinic space group $C2$ with $a = 135·2$ Å, $b = 112·1$ Å, $c = 187·0$ Å, $\beta = 94·6°$. They diffract to about 4 Å resolution.

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Glucosamine-6-phosphate Synthase from *Escherichia coli* Yields Two Proteins upon Limited Proteolysis: Identification of the Glutamine Amidohydrolase and 2R Ketose/Aldose Isomerase-Bearing Domains Based on Their Biochemical Properties

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The proteolysis of native glucosamine-6-phosphate synthase (M_r 67,000) from *Escherichia coli* was investigated using two nonspecific and five specific endoproteinases. α-chymotrypsin generated two nonoverlapping polypeptides CT_1 and CT_2 of M_r 40,000 and 27,000 lacking glucosamine-6P synthesizing activity. Amino terminal and carboxy terminal sequence analyses showed that cleavage occurred between positions 240 and 241 of the primary sequence without further degradation. The glutamine amidohydrolase activity was located in the CT_2 N-terminal polypeptide which was capable of incorporating 0.7 equivalent of the glutamine site-directed affinity label [2-^{3}H]-N^3-(4-methoxyfumaroyl)-diaminopropionic acid indicating that it bears the amidotransferase function. CT_1 which displayed a higher reactivity than CT_2 for fructose-6P binding contains the ketose/aldose isomerase activity. These data suggest the existence of a hinge structure essential for the catalytically efficient coupling between the ammonia generating domain and the sugar binding domain and support the model recently proposed by Mei and Zalkin in which *purF*-type amidotransferases contain a glutamine hydrolase domain of approximately 200 amino acids fused to an ammonia-transfer domain. © 1991 Academic Press, Inc.

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[2] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid; FMDP, N^3-(4-methoxyfumaroyl)diaminopropionic acid; [^{3}H]FMDP, racemic compound tritiated at the α amino acid position; Fru-6P, D-fructose-6-phosphate; GlmS, glucosamine-6P synthase; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T, trypsin; CT, chymotrypsin; BSA, bovine serum albumin; KCN, potassium cyanide.

Glucosamine-6P synthase (GlmS, L-glutamine:D-fructose-6P amidotransferase, EC 2.6.1.16) is a key enzyme in hexosamine biosynthesis (1). This homodimeric protein performs two coupled enzymatic reactions: the generation of ammonia from the amide nitrogen of glutamine and its subsequent transfer to the acceptor, fructose-6P, which undergoes an isomerization to generate glucosamine-6P. These properties mean that GlmS belongs to two classes of enzymes, the glutamine-dependent amidotransferases and the 2R ketose/aldose isomerases. The first part of the catalysis involves the participation of the amino terminal cysteine residue (2) which is believed to generate a γ-glutamylthiol ester intermediate and nascent ammonia (3). The second part of the reaction, that is formation of 2-amino-2-deoxy-glucose-6P, corresponding to the Heyns rearrangement (4), has been shown to require the abstraction of the C_1 pro-R hydrogen of a putative fructosimine-6P to form a transient *cis*-enolamine which, upon reprotonation at the *Re* face of carbon 2, gives rise to the product (5).

In spite of the fact that glucosamine synthase catalyses a slow glutamine hydrolyzing reaction (1% of V_{max}), we have been unable to detect any glucosamine-6P formation using exogeneous ammonia as an alternate nitrogen donor in the presence of fructose-6P and large amounts of enzyme. This fact, which is unique for GlmS since all the other glutamine-dependent amidotransferases can use exogeneous NH_3, might reflect a high shielding of the acceptor site together with an efficient channeling of the nascent ammonia in the presence of fructose-6P. In an effort to study the organization of the tertiary structure of the protein and to investigate a possible structural subdivision into catalytic and regulatory domains, we undertook a study of the susceptibility of GlmS toward controlled proteolysis.

MATERIALS AND METHODS

Materials. Chymotrypsin (EC 3.4.21.1.) from bovine pancreas (45 units/mg) was purchased from Serva. Endoproteinase Lys-C from *Lysobacter enzymogenes* (150 units/mg), endoproteinase Arg-C from submaxillary glands of mice (220 units/mg), Pronase from *Streptomyces griseus* (7 units/mg), aminopeptidase M (EC 3.4.11.2) from hog kidney (4 units/mg in 3.2 M ammonium sulfate) and carboxypeptidase Y (EC 3.4.16.1) from yeast (sequencing grade) were purchased from Boehringer Mannheim. TPCK-treated trypsin (EC 3.4.21.4) from bovine pancreas (200 units/mg) was from Cooper Biomedicals. Protease V8 (EC 3.4.21.19) from *Staphylococcus aureus* (540 units/mg) was from Miles Laboratory. Thermolysin (EC 3.4.24.4) type X from *Bacillus proteolyticus* (50–100 units/mg) was purchased from Sigma.

[U-^{14}C]fructose-6P (257 mCi/mmol) and U-[^{14}C]-L-glutamine (260 mCi/mmol) were obtained from CEA (Saclay, France); the synthesis of tritiated FMDP has been previously described (6). Glucosamine-6P synthase was obtained in pure form from *E. coli* NK 7356/pGM10$_A$ (7).

Activity assay. Colorimetric determination of glucosamine-6P and continuous monitoring of glutamate production were performed as previously described (2). The amidohydrolase activity of the native enzyme and of proteolytic fragments was analyzed using either L-glutamine (5 mM) or γ-L-glutamic p-nitroanilide (0.4 mM) as substrate in 100 mM phosphate buffer, pH 7.2, with high protein concentrations (0.3–0.8 μM). Glutamate production was quantified at 365 nm as described (2) and p-nitroaniline at 405 nm; under our conditions 1 A_{405} unit corresponded to the liberation of 100 nmol of the yellow chromophore (specific activity with this substrate, 0.063 U/mg).

Gel electrophoresis. Analytical polyacrylamide gel (12%) electrophoresis (PAGE) was performed in the presence of SDS according to Laemmli (8) using a Mini Protean II dual slab cell (Bio-Rad). Usually the gels were stained with Coomassie brilliant blue R-250 (9).

Limited proteolysis. Preliminary assays were conducted with 0.5 mg GlmS and 10 μg (2% w/w) proteolytic enzyme in 0.5 ml 50 mM phosphate buffer, 1 mM EDTA, pH 7.2, (buffer A) (except for thermolysin where EDTA was omitted) containing when indicated Fru-6P (10 mM) and/or L-glutamine (10 mM). After a 30-min incubation at 37°C, the reaction was stopped by addition of the appropriate inhibitor (4 mM PMSF (final concentration)—from a 100-mM stock solution in iPrOH—for trypsin, endoArg-C, Pronase, and chymotrypsin; 0.4 mM leupeptin for endoLys-C; 5 mM EGTA for thermolysin) and the sample was immediately analyzed by SDS–PAGE.

The chymotryptic digestion (1% w/w) in buffer A alone or containing L-glutamine (10 mM) or Fru-6P (10 mM) or both was followed by SDS–PAGE over a time period of 7 h; the enzyme activity remaining was determined by the coupled spectrophotometric assay.

For preparative purposes, 64 mg (49 A_{280} units) of GlmS was incubated in the presence of 640 μg chymotrypsin in 2.7 ml buffer A for 3 h. The reaction was quenched with PMSF and the mixture loaded on a 2.5 × 135 cm TSK HW50(S) (Merck) gel filtration column running at 50 ml/h in 100 mM phosphate buffer. The protein-containing fractions were analyzed by SDS–PAGE. CT$_1$ was further purified on Superose 12 (same buffer, 0.1 ml/min) to give 41 mg (65% yield) of the pure 40 kDa fragment. A similar 2-h digestion of 136 mg GlmS in buffer A containing glutamine (10 mM) gave after purification 35 mg CT$_1$ and 27 mg CT$_2$. Both fragments were stored at −80°C in buffer A containing 10% glycerol.

Active site labeling of proteolytic fragments. Native enzyme and each of the purified proteolytic fragments were separately incubated at 1 mg/ml in buffer A with 2 mM [^{3}H]FMDP for 12 h. The protein was purified by gel filtration and the specific radioactivity determined by scintillation counting. Since the exact correspondence between protein concentration and 280 nm absorbance was unknown for the trypsin (T) and chymotrypsin (CT$_1$ and CT$_2$) fragments (the correction factor is 1.3 for native enzyme), the stoichiometry of incorporation of radiolabeled substrate or inhibitor was calculated from the results of Bradford (10) protein determinations (BSA as a standard).

Labeling with fructose-6P. The protein under study was thoroughly dialyzed against buffer A and incubated at 2–4 mg/ml for 2 h at room temperature with 5 mM U-[^{14}C]-Fru-6P (6.36 × 10^6 cpm/μmol). The resulting solution was loaded at 2°C on a G-25 gel filtration column running at 15 ml/h in buffer A. The fractions (1 ml) kept on ice were analyzed for their 280-nm absorbance and their radioactivity determined on 40-μl aliquots. The protein containing fractions were pooled and the specific radioactivity determined. In the experiments using cyanide ions, KCN was added to 20 mM at the end of the incubation period; in this case, gel filtration was carried out at room temperature using Superose 12 in buffer A.

Amino terminal sequence and amino acid analysis. Edman degradation of the polypeptide fragments resulting from limited proteolysis was performed by Dr. Bonicel (CNRS-CBM, Marseille) using an Applied Biosystems Model 470A automated sequencer. Amino acid composition was determined by Ms. Guidoni (CNRS-CBM, Marseille) on a LKB analyzer following a 24-h hydrolysis in 5.7 N HCl containing 0.1% phenol.

Carboxy terminal sequence analysis. The carboxy terminal residue determinations were performed according to the protocol described by Klemm (11). The samples resulting from the digestion of 20 nmol protein in 200 μl 0.1 M pyridinium acetate, pH 5.6, containing 1% SDS and 0.1 mM norleucine were analyzed on an LKB amino acid analyzer after 1, 2, 5, 10, 20, 30, and 60 min digestion with 0.2 nmol carboxypeptidase Y.

RESULTS

Limited Proteolysis

Glucosamine-6P synthase (GlmS) from *E. coli* was subjected to controlled proteolysis at pH 7.2 for 0.5 h by five residue-specific endopeptidases (trypsin, proteinases endoArg-C and endoLys-C, protease V8 and chymotrypsin) and two nonspecific proteases, thermolysin and Pronase using a ratio of protein to proteinase of 50 to 1 in the absence or presence of the two substrates. As shown in Fig. 1A, only Lys-C, trypsin, chymotrypsin, and Pronase generated a limited number of fragments. Whereas trypsin and Pronase gave only one 40-kDa band, the two other proteases gave two similar-sized proteins of 40 and 30 kDa. The rate of proteolysis reactions appeared to be dependent on the endopeptidase used: overall, chymotrypsin digested GlmS the fastest since in the absence of added substrates, the reaction was practically complete within 30 min. The presence of both L-glutamine and Fru-6P exerted a marked protective effect against proteolysis (compare Figs. 1A and 1B) as exemplified in the case of chymotrypsin.

Affinity Labeling of the Proteolytic Fragments

Since protease treatment resulted in loss of glucosamine-6P synthesizing activity we used alternative methods to assess the catalytic functions of the clipped domains. We tested the behavior of the fragments resulting from trypsin (T) or chymotrypsin (CT$_1$, CT$_2$) digestion with the affinity label [^{3}H]FMDP (9240 cpm/nmol) which covalently derivatizes the amino terminal cysteine residue of the native proteine as a stable Michael adduct (12). Each fragment resulting from the digestion of 0.5 mg was incubated overnight with FMDP in excess (2 mM) and
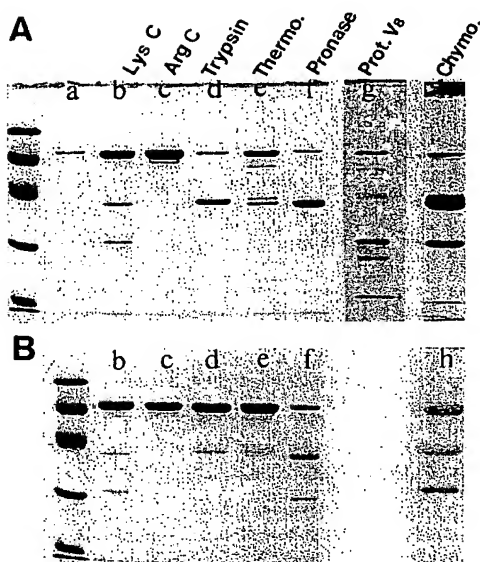
FIG. 1. SDS–PAGE (12% gel) analysis of the proteolytic digestion of GlmS from *E. coli* (1 mg/ml) with various proteinases (2% w/w) for 30 min at 37°C. (A) Experiments run in 50 mM phosphate buffer, 1 mM EDTA, pH 7.2 (buffer A). The following standards (left lane) were used: phosphorylase a (92 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lactalbumin (14.4 kDa). (B) experiments run in buffer A containing L-glutamine (10 mM) and Fructose-6P (10 mM).

separated from the small molecules by gel filtration. As shown in Table I, CT_2 incorporated per mole the same amount of tritium as the native enzyme corresponding to a stoichiometry of 0.7.

Proteolysis with Chymotrypsin

The time course of proteolysis by chymotrypsin (1% w/w) was followed under a different set of conditions. In buffer A alone, total disappearance of the starting enzyme and loss of the associated enzymatic activity occurred within less than 30 min of incubation at 37°C to generate CT_1 (40 kDa) as the main fragment (Fig. 2, inset a). In the presence of glutamine, the activity vs time profile was similar but the different pattern observed by SDS–PAGE (Fig. 2, inset b) suggested a slower proteolysis rate and a stabilization of CT_2 against further degradation. In the presence of Fru-6P the time-dependent loss of activity followed apparent first-order kinetics ($t_{1/2} = 1$ h) when analyzed as a semi-log plot (data not shown). Under these conditions the starting enzyme which disappeared over a 12-h period (Fig. 2, inset c) generated after about 3 h either a 80:20 mixture of CT_1 and CT_2 in the presence of glutamine (Fig. 2, inset d) or mainly CT_1 when glutamine was omitted (inset c).

Since the presence of fructose affected only the rate of native enzyme proteolysis without any noticeable effect on the fragment distribution, the conditions selected to isolate large amounts of the clipped proteins were the presence of glutamine but the absence of fructose-6P.

Characterization of the Chymotryptic Fragments

Edman degradation of the two fragments gave the results summarized in Table II. CT_2 corresponds to the amino terminal domain, whereas CT_1 begins at position 241 of the primary sequence. The amino acid analysis was in good agreement with the existence of a single proteolytic cleavage site. The molecular weight of each polypeptide, calculated from the results of this analysis by the Delaage method (13) gave 37.6 and 29.1 kDa the sum of which is similar to the native subunit molecular weight (67 kDa). These values would suggest a cleavage site around amino acid 270. Knowing the specificity of the protease employed gives Tyr^{240}, Tyr^{248}, Tyr^{251}, Tyr^{257}, and Tyr^{304} as possible candidate sites. The C-terminal analysis of CT_2 using carboxypeptidase Y identified Tyr^{240} as the cleavage site. The identification of glutamate as the first amino acid to be released by carboxypeptidase Y digestion of CT_1 definitively established that the only amide bond cleavage occurred between Tyr^{240} and Asp^{241} giving a 240 amino acid N-terminal domain ($M_r = 26,488$) and the corresponding 368 amino acid C-terminal domain ($M_r = 40,319$). (See Fig. 3).

Catalytic Activities Associated with the Two Domains

Glutamine binding site. The glutamine binding site fragment was further characterized by its amidohydrolase activity, that is, the capacity to hydrolyze L-glutamine into glutamate and ammonia. In the absence of Fru-6P, glucosamine-6P synthase catalyzes this reaction with a turnover of 4.2 min^{-1} per monomer corresponding to 0.8% of the normal reaction. As shown in Table III, only CT_2 is able to perform significant hydrolysis of glutamine albeit with a low efficiency. This property was confirmed

TABLE I

Stoichiometry of Fixation of [³H]FMDP on Purified Fragments Resulting from Proteolytic Treatment with Trypsin (T) and Chymotrypsin (CT_1, CT_2) in Buffer A Containing 10 mM Glutamine

Sample	Size (kDa) on SDS–PAGE	Recovered protein[a]		Stoichiometry[b]
		nmol[b]	cpm	
GlmS	70	2.2	14,190	0.70
T	40	3.3	3,560	0.12
CT_1	40	4.8	6,400	0.14
CT_2	30	3.7	22,940	0.67

[a] Following overnight incubation with 2 mM [³H]FMDP (9240 cpm/nmol) and gel filtration on Superose 12.

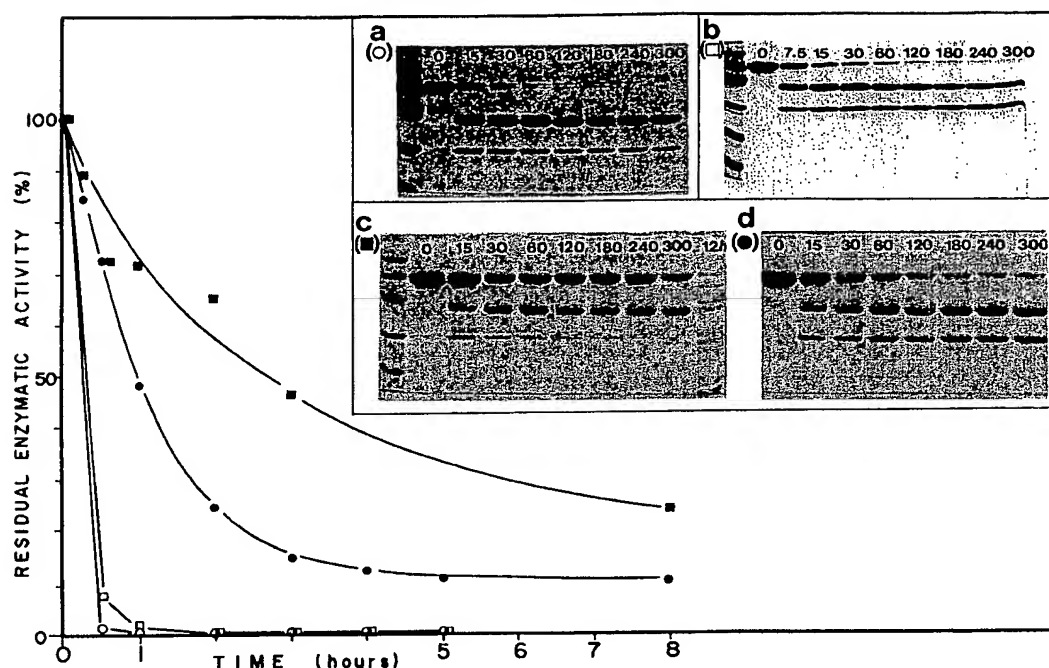[b] Determined by Bradford (10) analysis.

FIG. 2. Time course of the limited proteolysis of GlmS from *E. coli* (1 mg/ml) by α-chymotrypsin (1% w/w) in 50 mM phosphate buffer, 1 mM EDTA, pH 7.2 (buffer A) (O), in buffer A containing 10 mM L-glutamine (□), or 10 mM fructose-6P (■) or both (●) was monitored by the loss of catalytic activity. (Inset) SDS–PAGE analysis of reaction products (a, c, d: 12% gel; b, 15% gel). Left lane of each panel: standards as in Fig. 1. (a) proteolysis in buffer A, (b) in buffer A containing 10 mM L-glutamine, (c) in buffer A containing 10 mM fructose-6P, and (d) in buffer A containing 10 mM L-glutamine and 10 mM fructose-6P. Aliquots were removed at the time (minutes) indicated at the top of the gel and quenched with PMSF to 0.4 mM. In a control experiment GlmS activity was shown to be unaffected following a 7-h incubation at 37°C in the absence of proteolytic enzyme.

by the use of a substrate analog, γ-glutamyl-*p*-nitroanilide. This glutamate derivative can undergo an enzyme-catalyzed amide bond cleavage to generate, besides glutamate, *p*-nitroaniline which was monitored continuously at 405 nm (14). The turnover of this compound by native enzyme is about 1% of the catalysis and a similar activity could be detected with the small chymotryptic fragment CT_2. Additional characterization of the glutamine binding domain made use of the irreversible inhibitor FMDP. As described above (Table I), CT_2 was as efficient as the wild

type enzyme in the incorporation of the tritiated inhibitor and fivefold more efficient than CT_1.

Fructose-6P binding site. Incubation of extensively dialyzed GlmS in the presence of 20 mM U-[^{14}C]-Fru-6P for 2 h resulted, following gel filtration at 4°C, in the incorporation of 1 equivalent of sugar per enzyme monomer. Under the same conditions CT_1 incorporated 16% of this value, whereas the stoichiometry of substrate fixation to CT_2 was below 1.5%. When the same reaction was performed in the presence of 20 mM KCN to stabilize

TABLE II

Characterization of Glucosamine-6P Synthase Chymotryptic Fragments

| Fragment | Size (kDa) | | | NH$_2$ End | COOH End[a] |
	SDS–PAGE	Composition[b]	Prediction[c]		
CT_1	40	37.6	40.3	DAGDKIYRH	Glu
CT_2	30	29.1	26.5	CGIVGAIAQR	Tyr

[a] Determined from C-terminal analyses.
[b] Calculated from amino acid composition by the Delaage method (13).
[c] From primary sequence.

CT₁   CT₂

FIG. 3. SDS–PAGE analysis (12% gel) of the fragments isolated from the two preparative experiments described under Materials and Methods. The following markers (left lane) were used: phosphorylase a (92 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lactalbumin (14.4 kDa).

a putative imine intermediate GlmS:Fru-6P, the labeling of CT_1 did increase but there was a loss of selectivity since labeling of CT_2 also increased ($CT_1/CT_2 = 1.5$ vs 10 under the previous conditions).

DISCUSSION

The reaction catalyzed by glucosamine-6P synthase can be considered as two enzymatic steps and raised the question of the existence of two distinct domains responsible for the generation of ammonia from glutamine on the one hand and the conversion of a putative 2-fructosimine-6P into glucosamine-6P on the other hand. Proteolytic enzymes were used to probe the existence of such domains. Preliminary experiments with proteases of different specificity identified chymotrypsin as the enzyme of choice: it generated (Fig. 1) two fragments of 40 and 30 kDa, one of which covalently bound the tritiated affinity label FMDP with the stoichiometry of the native enzyme. Careful investigations on the conditions of chymotrypsin digestion were conducted to optimize the production of these fragments CT_1 and CT_2. The time course of the digestion (Fig. 2) revealed a very rapid loss in enzyme activity in the absence of added Fru-6P ($t_{1/2} \sim 10$ min). When present at 10 mM in the proteolysis reaction, Fru-6P increased the lifetime of GlmS which underwent first-order time-dependent loss of activity ($t_{1/2} = 3$ h, no glutamine present; $t_{1/2} = 1$ h, in the presence of 10 mM glutamine). In both cases the presence of glutamine in the mixture protected the small fragment against further degradation, suggesting its possible role in glutamine binding.

The preparative purification of CT_1 was carried out by incubation of large amounts of enzyme in phosphate buffer in the absence of the substrates (cf. Material and Methods); under these conditions the 40-kDa fragment was almost the only compound to be produced in good yield. The presence of glutamine in the reaction mixture slowed down the rapid digestion of the 30-kDa fragment (CT_2) allowing its purification as well as purification of CT_1. Yields were lower in this experiment because of the difficulty in separating native protein from the fragments in our gel filtration conditions (data not shown). The overall yield of these two experiments was nevertheless sufficient (75 mg CT_1 and 27 mg CT_2 from 200 mg GlmS) to further characterize the fragments. Based on results of amino acid analyses, the molecular weights calculated using the Delaage method (13) were in good agreement with the values deduced from SDS–PAGE calibration. Amino terminal analysis suggested a single cleavage site at Tyr[240]; carboxy terminal analysis confirmed this site and showed that no amino acids were missing between the two fragments.

No glucosamine-6P synthesizing activity could be recovered from a stoichiometric mixture of CT_1 and CT_2 even at high concentration (1 μM, 50-fold higher than the amount of enzyme that is used in the routine assay): this observation suggests that the tight association of the two domains in the native dimer is necessary to ensure an efficient transfer of nascent ammonia, generated at the glutamine binding site, to the fructose-6P acceptor site.

The identification of the two fragments as the amidotransferase and ketose/aldose isomerase-bearing domains required the demonstration of a catalytic activity characteristic of each function. The binding of the glutamine site-directed affinity label, FMDP, was used as the first criteria. The very efficient binding of this reagent to the amino terminal CT_2 suggested that FMDP covalently derivatized the amino terminal thiol group of the 240 amino acid polypeptide (there are no other cysteine in this fragment) as demonstrated in the native enzyme (2). However since CT_1 contains three other cysteine residues which

TABLE III

Catalytic Properties of GlmS Chymotryptic Fragments

Sample	Amidohydrolase activity min⁻¹ (%)		Stoichiometry of [¹⁴C]Fru-6P fixation[a] (%)	
	L-Gln	γ-Glu-pNO₂ anilide	No KCN	With KCN
GlmS	4.2 (100)	4.4 (100)	1.0 (100)	0.87 (100)
CT₁	0.02 (0.5)	0.064 (1.4)	0.16 (16)	0.62 (72)
CT₂	0.3 (7.1)	2.4 (56)	0.015 (1.5)	0.39 (45)

[a] Expressed as moles of fructose-6P bound per mole of native enzyme ($M_r = 70,000$) or per mole of proteolytic fragment (CT₁ = 40,000; CT₂ = 30,000).

might become more accessible to the reagent after separation of the two domains, it is not surprising that this fragment was also labeled somewhat after a 12-h incubation period.

In addition to its ability to bind covalently the affinity label FMDP, the small fragment also exhibited a weak glutamine aminohydrolase activity. The turnover of the reaction, although 20-fold higher than that detected for the other fragment was only 17% that of the native enzyme (Table III). When assayed with γ-glutamic p-nitroanilide, a substrate of γ-glutamyl transpeptidase (14), the specific activity of CT_2 was 120% that of GlmS (corresponding to a turnover of 56% that of native enzyme), suggesting a more favorable interaction between CT_2 and the aromatic substituent of the glutamine analogue than with glutamine itself.

Characterization of the 40-kDa fragment turned out to be more difficult. No glucosamine-6P formation could be detected upon incubation of CT_1 with Fru-6P and ammonia. This observation is, however, consistent with the fact that glucosamine-6P synthase is the only known glutamine-dependent amidotranferase not to use NH_3 as an alternate substrate to glutamine (2, 3). Since no affinity label was available at that time we made use of the discovered capacity of native protein to stoichiometrically bind U-[^{14}C]-Fru-6P. This observation is currently being explored to identify a possible lysine residue involved in imine formation with the aldehyde form of fructose-6P. As described in Table III, CT_1 binds 0.16 equivalent of this substrate, whereas CT_2 binds only one-tenth of this amount. Since high radioactive backgrounds resulted from treatment with tritiated sodium cyanoborohydride to stabilize the putative imine adduct, we preferred to use cyanide ions. These conditions have been used with success to prove the existence of a Schiff base in the mechanism of transaldolase from *Candida utilis* (15). Although KCN addition facilitated isolation of the radioactive complex which could now be performed at room temperature, it also significantly increased the labeling of CT_2. This result is not surprising, however, since working in irreversible conditions (formation of aminonitrile in the presence of CN^- in excess) favors unspecific labeling of NH_2 groups.

In conclusion, the demonstration of the existence of two structural domains as independently folding units is consistent with the association of two different proteins bearing, respectively, glutamine hydrolase and ketose/aldose isomerase activity and fits perfectly with the two-domain model proposed recently for the *purF*-type amidotransferases (16). It is interesting to note that of the 18 tyrosine residues present in the GlmS protein chymotrypsin attacks specifically and almost exclusively the Tyr[240] residue. This suggests that the residue is indeed part of the hinge between the two domains accessible from the outside while the other residues are protected in the

two presumably highly structurated amino and carboxy terminal domains. A similar dissociation of two activities acting in cooperation during the overall catalysis has been previously demonstrated by proteolysis of *Neurospora crassa* anthranilate synthase (17), a member of the *trpG* amidotransferase subfamily, and by controlled digestion of the *trpC* encoded N-(-5-phosphoribosyl)anthranilate isomerase:Indole glycerol phosphate synthase from *E. coli* (18). In both cases a specific enzymatic activity was associated with each purified fragment. As described above, no NH_3-dependent glucosamine-6P forming activity could be detected in the 40-kDa fructose-6P binding domain of GlmS; this result which corroborates the previously reported inability of the native enzyme to use ammonia as an alternate substrate suggests the loss of an NH_3 binding-site during the evolution of this glutamine-dependent amidotransferase gene. The identification of two non-overlapping domains will permit overexpression of both fragments suitable for physicochemical studies.

ACKNOWLEDGMENTS

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